Immunostimulatory and Antitumor Activities of Monoglycosylceramides Having Various Sugar Moieties

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Ten kinds of monoglycosylceramides (MonoCers), having the same ceramide portion and different sugar moieties, were synthesized and their immunostimulatory and antitumor activities were examined. The manner of combination between sugar and ceramide has been demonstrated to affect the manifestation of immunostimulatory and resultant antitumor activities of MonoCers, and in the case of D-MonoCers having the D-sugar, α-D-MonoCers (sugar combined to ceramide in an α-configuration) show stronger activities than β-D-MonoCers. Furthermore, the form of sugar, not the furanose-form but the pyranose-form, and the 2″- and 4″-hydroxyl groups of the pyranose-form of sugar, seemed to play an important role in the manifestation of the activities of α-D-MonoCers.

Key words agelasphin; monoglycosylceramide; immunostimulating agent; antitumor agent

Recently, galactosylceramide (GalCer) was identified as an essential component of the neural receptor for type 1 human immunodeficiency virus surface glycoprotein gp120,[1] and it has drawn considerable attention. The structural studies of monoglycosylated ceramides (MonoCers) such as GalCers and glucosylceramides (GluCers) isolated from organ tissues were carried out two decades ago, and it was demonstrated that they have β-MonoCer structures, *i.e.*, sugar combined to ceramide in a β-configuration.[2~4] In addition, various β-GalCers (galactose combined to ceramide in a β-configuration)[5,6] and β-GluCers[7~9] were isolated from marine organisms, although their *in vivo* antitumor activities have not been reported yet. By contrast, we found that agelasphins having α-GalCer structures (galactose combined to ceramide in an α-configuration) show strong antitumor activities against mice subcutaneously inoculated with murine melanoma B16 cells, and their potencies are stronger than those of the β-GluCer type of agelasphin.[10~13]

Furthermore, when we compared the antitumor activities of α-, β-GalCers and α-, β-GluCers, which have the same ceramide portion,[14] against mice subcutaneously implanted with murine melanoma B16 cells or fibrosarcoma Meth A cells, α-GalCer and α-GluCer showed stronger suppressive effects of tumor growth than their β-types.[15] These findings suggested that the manner of combination between sugar and ceramide greatly affects antitumor activities.

We were also interested in the different antitumor activities between α-GalCer and α-GluCer because the difference was caused only by a different configuration of the 4″-hydroxyl group of sugar moiety. Subsequently, the question arose as to whether other hydroxyl groups of the sugar portion also affect the antitumor activities of α-MonoCers. To address the question, we synthesized six additional kinds of MonoCers which have the same ceramide portion and different sugar moieties, and performed the following experiments using ten kinds of MonoCers.

In this paper, we describe that the manner of combination between sugar and ceramide greatly affects immunostimulatory and resultant antitumor activities of MonoCers, that not the furanose-form but the pyranose-form of sugar is important in the manifestation of the activities of α-D-MonoCers, and that the 2″- and 4″-hydroxyl groups of the pyranose-form of sugar play an important role in the activities of α-D-MonoCers.

MATERIALS AND METHODS

Animals Female C57BL/6 mice, BALB/c mice, and BDF_1 mice purchased from Nippon SLC Co., Ltd. were used in the experiments. Mice were reared under our standard laboratory conditions and given the standard diet and water *ad libitum*.

Syntheses and Physical Properties of MonoCers Ten kinds of MonoCers used in this paper were synthesized in our laboratory and Fig. 1 shows their structures. The synthetic procedures for AGL-517, AGL-562, AGL-563 and AGL-564 and their physical properties were previously reported.[14] AGL-569, AGL-571, AGL-574, AGL-576 and AGL-577 were synthesized in the general strategy as shown in Chart 1. In brief, their glycosyl donors (**3** and **4**) were derived from raw sugars (**2**) through four steps: methyl glycosidation, benzyl protection, hydrolysis of methyl glycoside, and then fluorination with DAST (diethylaminosulfur trifluoride–triethlamine complex). These glycosyl fluorides (**3** and **4**) were coupled[16] with a ceramide (**1**),[13] and the resulting α- and β-glycosides (**5** and **7**) were deprotected by hydrogenolysis to give corresponding MonoCers (**6** and **8**). AGL-575, having 2-deoxy-D-galactose in an α-anomeric configuration, was synthesized as shown in Chart 2. 2-Deoxy-D-galactose (**11**) was peracetylated followed by bromination, and then glycosylated[17] with a **1**. The resulting α-glycoside (**13**) was deprotected by methanolysis to give AGL-575. The structures of these six compounds were confirmed by ^{1}H-NMR and MS spectra using a JEOL GX-500 and JEOL SX-102, respectively. The physical properties of the six compounds are shown below.

AGL-569: $[\alpha]_D^{23} = +49.7°$ ($c = 0.35$, pyridine), mp 101.5.0—105 °C. FDMS *m/z*: 645 $(M+1)^+$. ^{1}H-NMR

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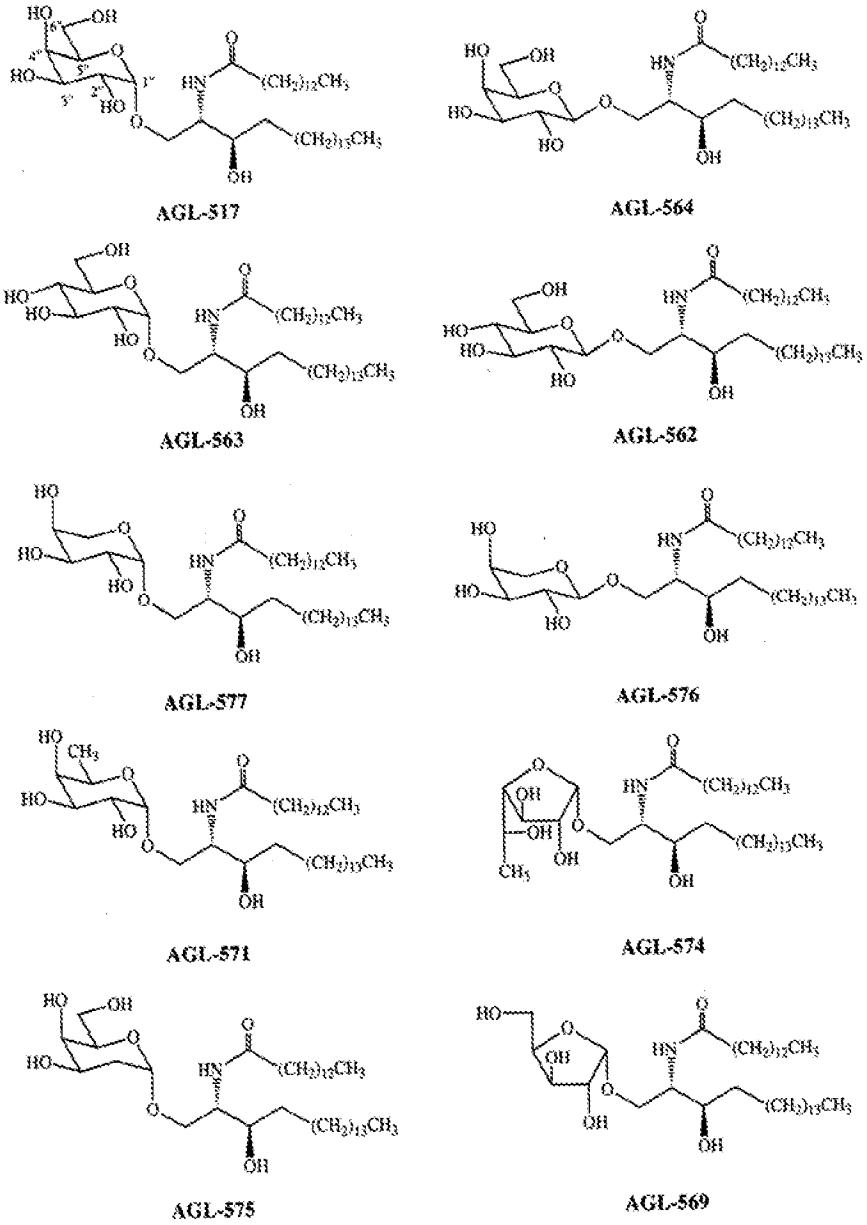
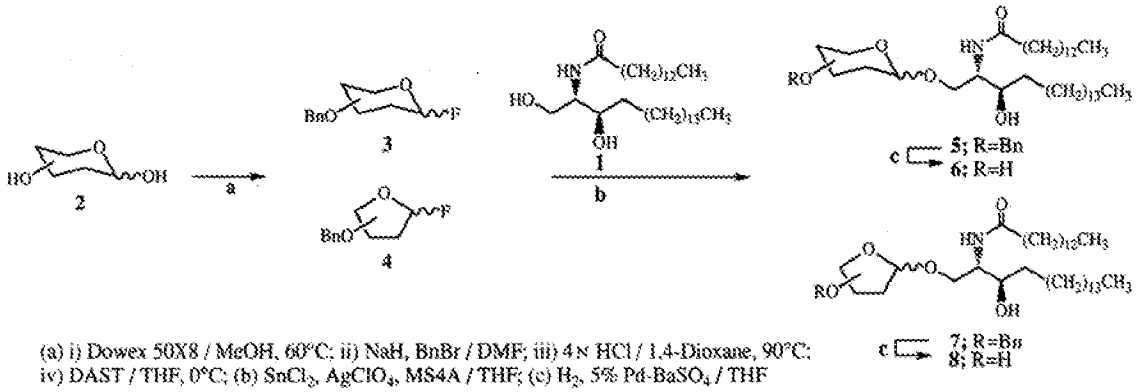
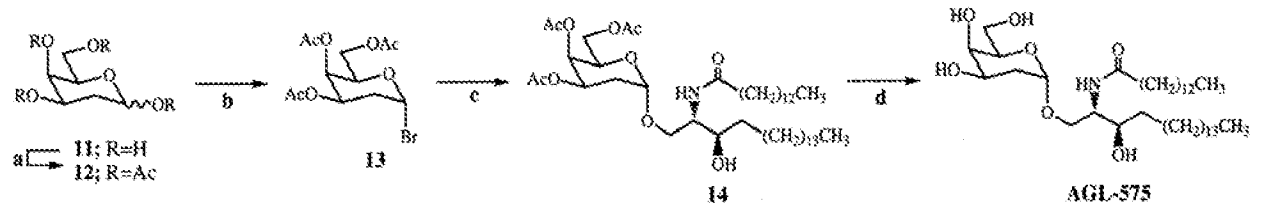


Fig. 1. Structures of MonoCers Employed in This Study



(a) i) Dowex 50X8 / MeOH, 60°C; ii) NaH, BnBr / DMF; iii) 4 N HCl / 1,4-Dioxane, 90°C; iv) DAST / THF, 0°C; (b) $SnCl_2$, $AgClO_4$, MS4A / THF; (c) H_2, 5% Pd-$BaSO_4$ / THF

Chart 1. Synthetic Procedures for AGL-569, AGL-571, AGL-574, AGL-576, and AGL-577



(a) Ac_2O / Pyr; (b) HBr / CH_2Cl_2; (c) **1**, Ag_2O, Et_4NBr / CH_2Cl_2 ; (d) NaOMe / MeOH

Chart 2. Synthetic Procedures for AGL-575

(500 MHz, C_5D_5N) δ (ppm): 8.42 (1H, d, J=8.6 Hz), 5.49 (1H, d, J=4.3 Hz), 4.81 (1H, m), 4.72 (2H, m), 4.46 (1H, dd, J=3.3, 10.1 Hz), 4.38 (1H, dd, J=4.3, 11.6 Hz), 4.32 (2H, m), 4.22 (1H, m), 2.42 (2H, t, J=7.3 Hz), 1.75—1.95 (5H, m), 1.54 (1H, m), 1.00—1.42 (44H, m), 0.88 (6H, t, J=7.0 Hz). *Anal.* Calcd for $C_{37}H_{73}NO_7$: C, 69.01; H, 11.43; N, 2.17. Found: C, 69.32; H, 11.63; N, 2.03.

AGL-571: $[\alpha]_D^{23}$ = +64.6° (c=1.0, pyridine). mp 143.0—144.5 °C. FDMS m/z: 659 $(M+1)^+$. ^{1}H-NMR (500 MHz, C_5D_5N) δ (ppm): 8.48 (1H, d, J=8.5 Hz), 5.36 (1H, d, J=3.7 Hz), 4.73 (1H, m), 4.57 (1H, m), 4.34—4.49 (3H, m), 4.23—4.30 (2H, m), 4.11 (1H, br s), 2.47 (2H, t, J=7.3 Hz), 1.80—1.94 (4H, m), 1.58 (1H, m), 1.55 (3H, d, J=6.7 Hz), 1.39 (2H, m), 1.19—1.33 (43H, m), 0.88 (6H, t, J=7.0 Hz). *Anal.* Calcd for $C_{38}H_{75}NO_7$: C, 69.36; H, 11.49; N, 2.13. Found: C, 69.52; H, 11.79; N, 1.92.

AGL-574: $[\alpha]_D^{23}$ = +33.3° (c=1.29, pyridine). mp 100.0—100.5 °C. FDMS m/z: 659 $(M+1)^+$. ^{1}H-NMR (500 MHz, C_5D_5N) δ (ppm): 8.51 (1H, d, J=8.5 Hz), 5.31 (1H, d, J=4.3 Hz), 4.85 (1H, t, J=7.9 Hz), 4.62—4.69 (2H, m), 4.41 (1H, dd, J=4.0, 10.1 Hz), 4.37 (1H, m), 4.33 (1H, dd, J=3.1, 9.8 Hz), 4.25 (1H, m), 4.18 (1H, dd, J=5.5, 6.7 Hz), 2.50 (2H, t, J=7.3 Hz), 1.79—1.94 (4H, m), 1.58 (3H, d, J=6.1 Hz), 1.51 (1H, m), 1.19—1.42 (45H, m), 0.88 (6H, t, J=6.7 Hz). *Anal.* Calcd for $C_{38}H_{75}NO_7$: C, 69.36; H, 11.49; N, 2.13. Found: C, 69.64; H, 11.77; N, 1.96.

AGL-575: $[\alpha]_D^{23}$ = +40.9° (c=1.63, pyridine). mp 133.0—134.0 °C. FDMS m/z: 659 $(M+1)^+$. ^{1}H-NMR (500 MHz, C_5D_5N) δ (ppm): 8.46 (1H, d, J=8.6 Hz), 5.26 (1H, br s), 4.75 (1H, m), 4.53 (1H, m), 4.35—4.49 (5H, m), 4.18—4.27 (2H, m), 2.45—2.55 (3H, m), 2.22 (1H, dd, J=4.9, 12.2 Hz), 1.82—2.00 (5H, m), 1.60 (1H, m), 1.10—1.45 (44H, m), 0.88 (6H, t, J=6.7 Hz). *Anal.* Calcd for $C_{38}H_{75}NO_7$: C, 69.36; H, 11.49; N, 2.13. Found: C, 69.68; H, 11.82; N, 1.99.

AGL-576: $[\alpha]_D^{24}$ = +6.7° (c=1.28, pyridine). mp 126.0—129.0 °C. FDMS m/z: 644 M^+. ^{1}H-NMR (500 MHz, C_5D_5N) δ (ppm): 8.30 (1H, d, J=8.5 Hz), 4.78 (1H, d, J=6.7 Hz), 4.75 (1H, m), 4.70 (1H, m), 4.45 (1H, t, J=7.6 Hz), 4.28—4.32 (2H, m), 4.10—4.20 (3H, m), 3.75 (1H, br d, J=11.0 Hz), 2.43 (2H, t, J=7.3 Hz), 1.78—1.93 (4H, m), 1.53 (1H, m), 1.20—1.41 (45H, m), 0.88 (6H, t, J=7.0 Hz). *Anal.* Calcd for $C_{37}H_{73}NO_7$: C, 69.01; H, 11.43; N, 2.17. Found: C, 69.27; H, 11.65; N, 1.93.

AGL-577: $[\alpha]_D^{24}$ = +65.7° (c=1.23, pyridine). mp 111.0—113.0 °C. FDMS m/z: 644 M^+. ^{1}H-NMR (500 MHz, C_5D_5N) δ (ppm): 8.45 (1H, d, J=8.6 Hz), 5.40 (1H, d, J=3.1 Hz), 4.71 (1H, m), 4.59 (1H, dd, J=3.4, 9.5 Hz), 4.44 (2H, m), 4.33 (1H, br s), 4.20—4.28 (3H, m), 4.06 (1H, dd, J=2.1, 11.9 Hz), 2.45 (2H, t, J=7.3 Hz), 1.80—1.93 (4H, m), 1.56 (1H, m), 1.19—1.42 (45H, m), 0.88 (6H, t, J=6.7 Hz). *Anal.* Calcd for $C_{37}H_{73}NO_7$: C, 69.01; H, 11.43; N, 2.17. Found: C, 69.38; H, 11.59; N, 2.01.

Preparation of Spleen Cells Mice were weighed and sacrificed, the spleens were dissociated in 10% fetal calf serum (FCS, Gibco) RPMI 1640 (Gibco), and red blood cells were lysed with Tris NH_4Cl. The cells were washed three times using phosphate buffered saline (Nissui Pharmaceutical Co., Ltd.), and viable cells were counted and resuspended in 10% FCS RPMI 1640. These spleen cells were used for the following allogeneic mixed leukocyte reaction (MLR).

Allogeneic MLR Assays Spleen cells (1×10^5 cells/50 μl/well) from BALB/c mice (responder cells) and the same number of Mitomycin C-treated (50 μg/ml, 30 min) spleen cells from C57BL/6 mice (stimulator cells) suspended in 10% FCS RPMI 1640 medium were plated on a 96-well plate in triplicate. At the same time, various concentrations of MonoCers (10 μl/well) were added into each well, and the cell suspension was cultured at 37 °C, 5% CO_2 for 2 d. Then, 0.5 μCi/well of tritium-thymidine ([^{3}H]TdR) was added into each well, and 6 h later, the [^{3}H]TdR uptake into the cells was measured by a liquid scintillation counter.[18]

Tumor Growth Inhibitory Effects of MonoCers on Mice Inoculated with B16 Cells Subcutaneously Six female BDF_1 mice per group were used. B16 cells (1×10^6 cells/mouse) were subcutaneously inoculated into the mice on day 0. MonoCers (100 μg/kg) were intravenously administered on days 1, 5 and 9. Each tumor volume (length × width × height/2, mm^3) per mouse was measured using callipers on days 9, 12, 16 and 20.

Statistical Analysis Statistical analysis was done by a two-sided unpaired Student's t-test.

RESULTS AND DISCUSSION

To confirm the suggestion that the manner of combination between sugar and the ceramide of GalCer and GluCer greatly affects antitumor activities, we previously compared the antitumor activities of AGL-517 (α-GalCer), AGL-564 (β-GalCer), AGL-563 (α-GluCer), and AGL-562 (β-GluCer) against mice subcutaneously inoculated with murine melanoma B16 cells or murine fibrosarcoma Meth A cells, and found that AGL-517 and AGL-563 showed stronger antitumor activities than AGL-564 and AGL-562, respectively.[15] This demonstrated that concerning GalCers and GluCers which have the same

ceramide moiety, α-types show stronger antitumor activities than their β-types.

Since it had been suggested that agelasphins having α-GalCer structures show antitumor effects through the activation of immune systems,[13] to examine the relationship between antitumor activities and immunostimulatory activities of MonoCers we performed allogeneic MLR which is an assay to evaluate immune response using four MonoCers. As shown in Fig. 2, AGL-517 significantly stimulated lymphocytic proliferation at concentrations from 1 to 100 ng/ml, and at a concentration of 100 ng/ml, AGL-517 and AGL-563 showed significantly stronger lymphocytic proliferation stimulatory effects than AGL-564 and AGL-562, respectively. Their lymphocytic proliferation stimulatory effects on allogeneic MLR parallel their antitumor activities against tumor-bearing mice.[15] Taken together, the manner of combination between sugar and ceramide of GalCers and GluCers has been demonstrated to play an important role in their immunostimulating and resultant antitumor activities.

Furthermore, to examine whether the findings apply to other MonoCers having a sugar different from galactose and glucose, we synthesized AGL-576 (α-L-arabinosylceramide, α-L-AraCer) and AGL-577 (β-L-AraCer) (Fig. 1). Here, the manner of combination between sugar and ceramide of AGL-577 is the same as those of AGL-517 (α-D-GalCer) and AGL-563 (α-D-GluCer). When we compared the lymphocytic proliferation stimulatory effects of AGL-576 and AGL-577 on allogeneic MLR, AGL-577 stimulated the proliferation of lymphocytes in a concentration-dependent manner, and its potency was significantly stronger than that of AGL-576 at concentrations of 10 and 100 ng/ml (Fig. 3). This demonstrated that the manner of combination between sugar and ceramide greatly affects the immunostimulatory activities of MonoCers, and the α-configuration in the case of D-MonoCers, such as D-GalCer and D-GluCer, and the β-configuration in the case of L-MonoCers such as L-AraCer plays an important role in the manifestation of their strong immunostimulatory activities.

Here, we were interested in the finding that the lymphocytic proliferation stimulatory activity of α-D-GalCer (AGL-517) is significantly stronger than that of α-D-GluCer (AGL-563) (Fig. 2). Since the difference between AGL-517 and AGL-563 lies only in the different configuration of the 4″-hydroxyl group of the sugar moiety, it was suggested that the 4″-hydroxyl group plays an important role in the immunostimulatory activities of α-D-MonoCers. In contrast, data shown in Fig. 3 suggested that the 6″-hydroxyl group of the sugar moiety does not play an important role in the immunostimulatory activities. Then, to examine the roles of hydroxyl groups in the sugar portion of α-D-MonoCers regarding immunostimulatory activities, we synthesized AGL-571 and AGL-575, which have the pyranose-form of sugar lacking the 6″- and 2″-hydroxyl group, respectively. At the same time, we also synthesized AGL-574 and AGL-569 having the furanose-form of sugar to determine the role of the sugar form of α-D-MonoCers in immunostimulatory activities. AGL-571 markedly stimulated the proliferation of lymphocytes on allogeneic MLR in a concentration-dependent manner, and its potency was significantly stronger than that of AGL-575, AGL-574, or AGL-569 (Fig. 4). This demonstrated that not the furanose-form but the pyranose-form of sugar is important in the manifestation of immunostimulatory activities of α-D-MonoCers and that the 2″-hydroxyl group of the pyranose-form of sugar plays an important role in their immunostimulatory effects. In addition, we confirmed that the 6″-hydroxyl group does not greatly affect the activities.

Furthermore, to confirm that the immunostimulatory

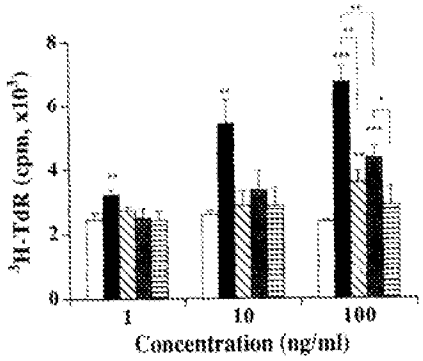


Fig. 2. Lymphocytic Proliferation Stimulatory Effects of AGL-517, AGL-564, AGL-563, and AGL-562 on Allogeneic MLR

Each value shows the mean ± S.D. ($n=3$). **, $p<0.01$; ***, $p<0.001$ (compared with vehicle treated group). *, $p<0.05$; **, $p<0.01$. □, vehicle; ■, AGL-517; ▨, AGL-564; ▩, AGL-563; ▥, AGL-562.

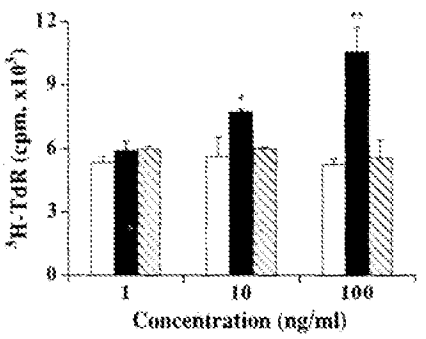


Fig. 3. Lymphocytic Proliferation Stimulatory Effects of AGL-577 and AGL-576 on Allogeneic MLR

Each value shows the mean ± S.D. ($n=3$). *, $p<0.05$; **, $p<0.01$ (compared with vehicle treated group). □, vehicle; ■, AGL-577; ▨, AGL-576.

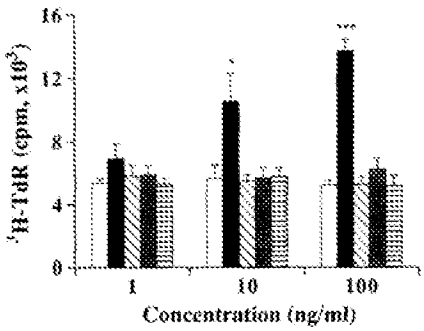


Fig. 4. Lymphocytic Proliferation Stimulatory Effects of AGL-571, AGL-575, AGL-569, and AGL-574 on Allogeneic MLR

Each value shows the mean ± S.D. ($n=3$). *, $p<0.05$; ***, $p<0.001$ (compared with the other groups). □, vehicle; ■, AGL-571; ▨, AGL-575; ▩, AGL-569; ▥, AGL-574.

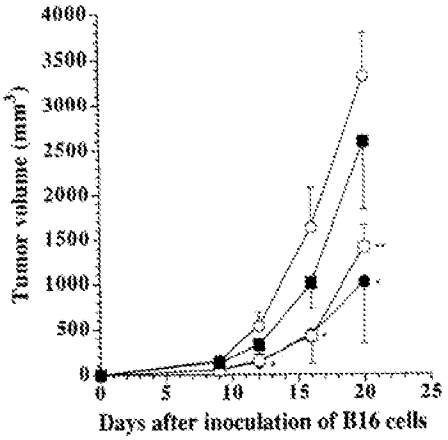


Fig. 5. Tumor Growth Inhibitory Effects of AGL-517, AGL-571, and AGL-575 on Mice Subcutaneously Inoculated with B16 Cells

B16 cells (1×10^6 cells/mouse) were subcutaneously inoculated into BDF_1 mice on day 0. 100 μg/kg of MonoCers were intravenously administered on days 1, 5 and 9. Tumor volume of each mouse was measured on days 9, 12, 16 and 20. Each value shows the mean ± S.E. *, $p < 0.05$; **, $p < 0.01$ (compared with control group). ○, control; ●, AGL-517; □, AGL-571; ■, AGL-575.

activities of the 2″ or 6″-deoxy type of α-D-MonoCers parallel their antitumor activities, we examined the tumor growth inhibitory activities of AGL-517, AGL-571 and AGL-575 against mice subcutaneously inoculated with B16 cells. As Fig. 5 shows, AGL-517 and AGL-571 significantly suppressed tumor growth, and their potencies were stronger than that of AGL-575. These findings resembled their immunostimulating activities.

Taken together, these findings suggest that the manner of combination between sugar and ceramide greatly affects immunostimulatory and resultant antitumor activities of MonoCers, that the α-configuration of D-MonoCers and the β-configuration of L-MonoCers play an important role in the activities, and that not the furanose-form but the pyranose-form of sugar and the 2″- and 4″-hydroxyl groups of the pyranose-form of sugar play an important role in the manifestation of the activities of α-D-MonoCers.

It is interesting that α-D-MonoCers such as α-D-GluCer and α-D-GalCer are considered not to exist in organ tissues, yet they show stronger immunostimulatory and resultant antitumor activities than β-D-MonoCers such as β-D-GluCer and β-D-GalCer, which are known to exist in organ tissues.[2—4]

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